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Translocation of the precursor of α-amylase into *Bacillus subtilis* membrane vesicles

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Bacilli vigorously secrete proteins into the extracellular environment, and are therefore used in industry for the bulk production of enzymes such as proteinases and amylases. Studies on the mechanism of protein translocation in these Gram-positive bacteria have been hampered by the lack of an *in vitro* system. To establish such a system for *Bacillus subtilis*, everted membranes were isolated from a strain deficient in the alkaline and neutral protease. Translocation-competent membrane vesicles were obtained only when a broad range proteinase-inhibitor cocktail was used during membrane isolation. This method efficiently prevented proteolysis of SecY, one of the core integral membrane components of the preprotein *translocase*. Translocation of the urea-denatured precursor of the *Bacillus licheniformis* α-amylase, preAmyL, and *B. subtilis* alkaline phosphatase, prePhoB, into the *B. subtilis* membrane vesicles require the *B. subtilis* SecA protein and are driven by ATP hydrolysis and the proton-motive force. These studies establish an authentic *in vitro* translocation system for protein secretion in *B. subtilis*.

Keywords: *Bacillus subtilis*; protein translocation; translocation; membrane.

In *Escherichia coli* and other Gram-negative bacteria, the outer membrane is the main barrier for protein export into the external medium. Proteins can be excreted in the medium either via a two-step reaction that involves separate translocation steps across the inner and outer membrane with the accumulation of a periplasmic intermediate [1], or directly across both the inner and outer membrane via specific secretory systems without the accumulation of a periplasmic intermediate. In the two-step reaction, proteins are first translocated as precursors across the inner membrane via the general secretory system, while outer-membrane translocation either occurs via a specific secretory system, or via a self-assembling outer-membrane-inserting subdomain that is proteolytically removed after the protein has crossed the outer membrane. In all these cases, the secretory system is rather specific for a particular (subset of) protein(s), while the general protein secretion system, the 'Sec-system', translocates proteins only into the periplasm. In contrast, Gram-positive bacteria lack an outer membrane and the secretory proteins are, therefore, immediately released into the external medium once they have passed the inner membrane. Although the cell wall may act as a molecular sieve and retard protein export by ionic interactions, folded exported proteins, in general, are able to pass this barrier. For this reason, Gram-positive bacteria, in particular Bacilli, are ideal hosts for the industrial production of bulk secretory enzymes.

Bacilli are known to vigorously secrete hydrolytic and proteolytic proteins, in particular at the late log phase. However, despite their great potential and commercial utilisation for protein export, little is known about the protein-translocation system in compared to the system in *E. coli* [2, 3]. *Bacillus* secretory proteins bear an amino-terminal signal sequence with a domain structure that is, to a large extent, similar to that of the *E. coli* signal sequences. Their signal sequences tend to be longer (by 5−7 amino acids) than those of *E. coli* [4]. These extensions occur in all three regions (n, h and c regions). The amino-terminus (n region) usually contains a higher instance of lysyl and arginyl residues, while the hydrophobic core (h region) is also longer than the corresponding region of *E. coli* signal sequences. The longer c region may reflect differences in substrate specificity of the Gram-positive signal peptidases as compared to the *E. coli* signal peptidase I, as cleavage occurs preferentially 7−9 residues or 3−7 residues, respectively, from the carboxyl-terminus of the h region [5]. Nevertheless, protein secretion across the membrane of *B. subtilis* is thought to be catalysed by a system that is homologous to the *E. coli* preprotein translocase [6]. In *E. coli*, preprotein translocation is mediated by a cytosolic chaperone, SecB, the translocation ATPase, SecA, and a large integral membrane protein complex with SecY, SecE, SecG, SecD and SecF as subunits [7]. Only SecA, SecE and SecY are essential components, whereas the other proteins add to the fidelity, and possibly to the specificity of the reaction. Many of the essential subunits of preprotein translocase of *B. subtilis* have been identified genetically. SecA is encoded by the *divA* gene [8, 9], and was originally found in a set of mutants conditionally defective in division or unable to sporulate. The integral membrane proteins SecY [10−12] and SecE [13] were identified after nucleotide sequence analysis of the chromosomal regions that contain the ribosomal *spc* operon and *nusG* that, in *E. coli*, contain *secY* and *secE*, respectively. The complete sequence analysis of the *B. subtilis* chromosome has also revealed the presence of homologues of SecD and SecF, but not of SecB [14]. *B. subtilis* contains not one but multiple signal peptidases that appear to be involved in the modulation of specificity towards different secretory proteins [15]. Finally, *in vivo* studies have

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Abbreviations. Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; $CF_3OPhzC(CN)_2$, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

established that, in analogy to *E. coli*, protein export in *B. subtilis* requires ATP and the proton motive force as energy sources [16, 17].

In addition to the large similarity of the secretory apparatuses of *B. subtilis* and *E. coli*, as paradigms of Gram-positive and negative bacteria, there are also some marked differences. PrsA, a protein that is membrane bound through the presence of an amino-terminal fatty acyl anchor, and itself is a secretory protein, has a profound effect on the secretion of some proteins in *B. subtilis* but is absent in *E. coli* [18, 19]. PrsA is thought to function as a prolyl-peptidyl isomerase, but *in vitro* its activity has not yet been demonstrated. Another point of interest is the degree of host specificity of the components of the secretory apparatus. The *E. coli* SecA cannot complement the *B. subtilis divA* mutant [20] whereas, under specific sets of conditions, the *B. subtilis* SecA can complement the conditionally lethal *secA* mutations [21−24]. Also, the *E. coli* and *B. subtilis* SecY proteins do not appear to be exchangeable [10]. Therefore, a comparison of the *E. coli* and *B. subtilis* secretory systems may reveal salient details on host and substrate specificity. Even with the wealth of *in vivo* and *in vitro* information available on the *E. coli* system, the differences and incompatibilities justify further studies of the mechanism of protein export in *B. subtilis*.

Over the last years, the advance in our knowledge on protein secretion in *E. coli* has been the results of integrated genetic, biochemical and enzymological investigations. A major advance has been the development of an *in vitro* protein translocation system based on everted inner membrane vesicles [25] and, more recently, by the use of purified preproteins and translocase components reconstituted into liposomes [26, 27]. Despite many attempts [28], it has not yet been possible to obtain a reliable *in vitro* translocation system for *B. subtilis*. This limits the possibilities to investigate the mechanism of protein export in this organism. For the Gram-positive organism *Staphylococcus carnosus*, translocation into isolated membrane vesiclesof a radiolabeled precursor protein following *in vitro* transcription-translation has been reported [29]. This system relies on the use of S-30 cytosolic extracts that contain a multitude of cytosolic proteins and factors that may contribute in an unspecified fashion to the translocation reaction. We now report on the development of an *in vitro* translocation system for *B. subtilis* using isolated membrane vesicles of a proteinase-deficient strain, purified cytosolic molecular chaperones, and the purified precursors of the *Bacillus licheniformis* α-amylase and *B. subtilis* alkaline phosphatase. This system enables biochemical studies of the mechanism and specificity of protein secretion in the Gram-positive Bacilli.

MATERIALS AND METHODS

Bacterial strains and growth media. Strains were grown in Luria-Bertani broth or on Luria-Bertani agar unless indicated otherwise. For plasmid bearing *E. coli* strains, the medium was supplemented with 50 μg/ml ampicillin and 0.5% glucose. Construction of vectors and overproduction of preAmyL was in *E. coli* DH5α (*supE44*, *ΔlacU169*, (*ϕ80lacZΔM15*), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*). Membrane vesicles were prepared from *B. subtilis* DB104 (*nprE18*, *aprEΔ3*) [30].

Preparation of secretion components and antibodies. *B. subtilis* [31] and *E. coli* SecA [32, 33] and *E. coli* SecB [34] were isolated from overproducing strains as described. The *B. subtilis* GroESL complex was isolated from *E. coli* cells harboring plasmid pREP9ESL [35]. *B. licheniformis* anti-(α-amylase) polyclonal antibodies were kindly provided by C. Lund-Jensen and S. Jørgensen (Novo Nordisk, Copenhagen, Denmark). The mAb directed against the His_6 tag was from Dianova. A polyclonal Ab directed against a synthetic peptide corresponding to amino acid residues 417−431 of *B. subtilis* SecY (ESQLVKRNYRGFMKN) was raised by Research Genetics. Complete™ protease inhibitor cocktail was purchased from Boehringer. All chemicals were from Sigma unless stated otherwise.

Construction of His_6-tagged preAmyL and prePhoB. A vector harboring a His_6 tag was generated by ligating an oligonucleotide linker into *Nco*I-digested pTRC99a [36] yielding pET302 [37]. The *B. licheniformis* α-amylase gene was isolated from plasmid pCJ199 (Lund-Jensen, C., unpublished results) as a *Bsp*HI−*Hin*dIII cassette, and ligated into *Nco*I−*Hin*dIII-digested pET302. The resulting vector, pET436, harbors the *amyL* gene in-frame with the His_6 tag under the control of the *Trc* promoter. In the case of prePhoB, the gene was amplified by PCR from the chromosome of *B. subtilis* DB104 as an *Nco*I−*Bam*HI fragment, and cloned into pET302, yielding pET461.

Expression and labelling of precursor proteins. An overnight culture of *E. coli* cells transformed with pET436 or pET461 was diluted 100-fold into fresh medium and grown aerobically. At mid-log phase, expression was induced with 1 mM isopropylthio-β-D-galactopyranoside and secretion of preproteins was inhibited by addition of 1 mM sodium azide [38]. After 2 h, cells were harvested by centrifugation and resuspended in cold 150 mM NaCl, 50 mM TrisCl, pH 7.5 (NaCl/Tris). Cells were broken by passing the suspension three times through a French pressure cell at 965 kPa. Inclusion bodies containing preAmyL were collected by centrifugation for 10 min at 10000×*g*, washed once with NaCl/Tris and dissolved in 6 M urea, 150 mM NaCl, 50 mM TrisCl, pH 7.5 (solution A). Debris and membrane fractions were removed by centrifugation for 60 min at 135000×*g*. The supernatant was loaded on a HiTrap Ni^+-nitrilotriacetate affinity column (Pharmacia) previously equilibrated with 1 column volume of 100 mM NiCl and 10 volumes solution A. Contaminating proteins were washed from the column by a 0−80-mM (preAmyL) or 0−60-mM (prePhoB) imidazole gradient in solution A, followed by elution of the precursor protein at 500 mM imidazole. The purity of the eluted preAmyL and prePhoB was confirmed by 10% SDS/PAGE [39] and blotting onto poly(vinylidene difluoride) membranes (Millipore) using a semi-dry blotting apparatus (BioRad). Immunodetection was carried out with a polyclonal Ab raised against α-amylase or with an anti-His_6 tag mAb. Blots were developed with a chemiluminescence kit (Tropix).

Purified protein was labelled with carrier free ^{125}I (Radiochemical Centre) according to the following procedure: residual imidazole was removed by chromatography on a PD-10 Sephadex column (Pharmacia) using solution A. Precursor potein (100 μg; 1 mg/ml) was transferred to a Iodo-Gen-(Pierce Rockford)-coated reaction vial, and the labelling reaction was started by the addition of 200 μCi $K^{125}I$. After 15 min incubation at 20 °C, the reaction was terminated by transferring the mixture to a new vial containing dithiothreitol at 10 mM final concentration. Free iodine was removed by chromatography on a PD-10 Sephadex column which had been washed with solution A, and the protein was stored at −80 °C in solution A.

Isolation of everted membrane vesicles. *B. subtilis* DB104 cells were grown aerobically to mid-exponential phase. Cells were harvested by centrifugation and resuspended in 50 mM potassium phosphate, pH 7.5, supplemented with Complete™ protease inhibitor cocktail according to the recommendations of the manufacturer. Everted membrane vesicles [28, 40] were prepared by passing the cell suspension through a French pressure cell three times at 965 kPa. Remaining cells and debris were removed by centrifugation for 10 min at 8000×*g*, and the membranes were collected from the supernatant by centrifugation for

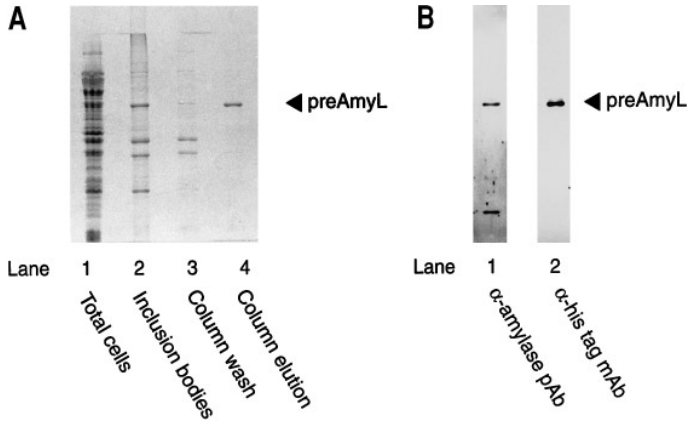

Fig. 1. Purification of His_6-tagged preAmyL. (A) Coomassie-blue stained SDS/PAGE of the cell lysate of *E. coli* DH5*α* harboring plasmid pET436 and induced for preAmyL expression (lane 1), isolated inclusion bodies (lane 2), the Ni^+-nitrilotriacetate affinity column chromatography fraction of the wash (lane 3) and elution with 500-mM imidazole (lane 4). The position of preAmyL is indicated. (B) Immunoblots of 500 mM-imidazole-eluted fraction developed with a polyclonal Ab directed against *α*-amylase and a mAb directed against the His_6 tag.

60 min at 135 000×*g*. Membrane vesicles were resuspended in 50 mM potassium phosphate, pH 7.5, at a protein concentration of 25 mg/ml, and stored in liquid nitrogen. The integrity of SecY and SecA in the membranes was verified by SDS/PAGE and immunoblotting using antibodies directed against SecA [31] and a SecY synthetic peptide. Where indicated, membranes were incubated with 6 M urea, 50 mM potassium phosphate, pH 7.5, for 1 h on ice, washed three times with 50 mM potassium phosphate, pH 7.5, and stored in liquid nitrogen. For ATPase measurements, membrane vesicles were prepared in 50 mM TrisCl, pH 7.5, instead of potassium phosphate. Membranes were incubated with 6 M urea, 50 mM TrisCl, pH 7.5, for 1 h on ice, washed three times with 50 mM TrisCl, pH 7.5, and stored in liquid nitrogen.

***In vitro* translocation and translocation ATPase assays.** *In vitro* translocation of ^{125}I-preAmyL and ^{125}I-prePhoB into *B. subtilis* membrane vesicles was essentially carried out as described for *E. coli* [33]. Reaction mixtures (50 µl) contained 50 mM Hepes/KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml BSA, 2 mM dithiothreitol, 2 mM MgOAc, membrane vesicles (25 µg protein), 10 mM creatine phosphate and 0.5 µg creatine kinase. When indicated, 0.5 µg purified *B. subtilis* or *E. coli* SecA protein, 1.6 µg purified *E. coli* SecB or 20 µg purified *B. subtilis* GroESL were added, followed by the addition of 2 µM (or when indicated 2 mM) ATP. Reactions were started by the addition of 1 µl ^{125}I-labelled precursor protein (typically 100 000 cpm; 0.5 µg protein). After 30 min at 37 °C, samples were chilled on ice, treated with proteinase K (0.5 mg/ml) for 15 min, precipitated with 7.5% (mass/vol.) trichloroacetic acid, washed with acetone, dissolved in SDS/PAGE sample buffer, and analysed by 10% SDS/PAGE and autoradiography. Translocation ATPase activity of urea-treated membrane vesicles was measured with preAmyL (10 µg/ml) and *B. subtilis* SecA (10 µg/ml) as described [41].

Analytical methods. Protein concentrations were determined by the method of Lowry [42] using BSA as a standard.

RESULTS

Isolation and purification of pre-*α*-amylase. To develop an *in vitro* translocation system for the analysis of protein export in *B. subtilis*, the precursor of the *B. licheniformis* *α*-amylase (preAmyL) was isolated. This protein is efficiently secreted by various *Bacillus* species [43, 44]. A His_6-tag was fused to the amino-terminus of preAmyL to facilitate purification of the precursor form. The gene was placed under control of an isopropylthio-*β*-D-galactopyranoside-inducible promoter of the *E. coli* pTRC99a expression vector. Under conditions where protein secretion in *E. coli* was blocked by the presence of 1 mM azide, large amounts of preAmyL accumulated in inclusion bodies. When the induction was preformed in the absence of azide, preAmyL was processed and secreted as detected by halo formation on starch-containing media (data not shown). The inclusion bodies were extracted with 6 M urea, and the denatured His_6-tagged preAmyL was purified by Ni^+-nitrilotriacetate affinity chromatography (Fig. 1A). The protein eluted from the column at an imidazole concentration above 100 mM, whereas most contaminants, including the mature AmyL, eluted from the column at lower imidazole concentrations (Fig. 1A). The presence of the His_6-tag and, thereby, the signal peptide, was confirmed by Western-blot analysis using a polyclonal Ab directed against AmyL (Fig. 1B, lane 1) and a mAb directed against the His_6-tag (lane 2). The same method was used to purify the precursor of *B. subtilis* alkaline phosphatase, prePhoB (Fig. 4B, lane 3). Since mature proteins are absent, this method efficiently allows for the purification of urea-denatured precursors to near homogeneity in a single step.

Protease inhibitors are needed for isolation of membrane vesicles containing intact SecY. *B. subtilis* produces many extracellular proteinases which may pose a major problem for the isolation of membrane vesicles competent of preprotein translocation. To reduce the protease activity, membranes were isolated from *B. subtilis* strain DB104 that lacks the alkaline proteinase and that is deficient in the neutral proteinase [30]. These combined mutations lead to a protease activity that is reduced to only 2.5% of the wild-type levels. In addition, the isolation method was optimised for speed. After harvesting, cells were subjected to French pressure cell treatment and membranes were collected by differential centrifugation. After sedimentation of unbroken cells and wall material, membranes were recovered from the supernatant by high-speed centrifugation. The integrity of SecY and SecA was determined by SDS/PAGE and immunoblotting. Although the total preparation time was less than three hours, and despite the use of strain DB104, SecY was completely degraded while SecA appeared stable (Fig. 2, left panel). The presence of Complete™, a protease inhibitor cocktail, throughout the membrane vesicle preparation reduced the proteolysis of SecY drastically (Fig. 2, right panel). When the Marburg strain 168 that produces wild-type levels of extracellular proteinases was used, it was not possible to obtain membrane

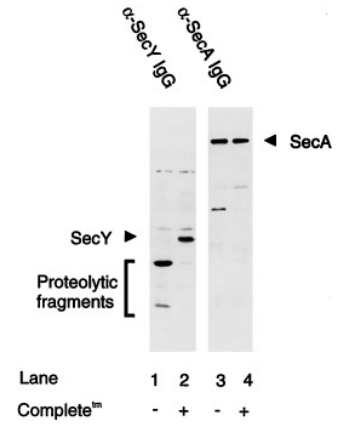

Fig. 2. Stability of SecY and SecA in *B. subtilis* DB104 membrane vesicles. *B. subtilis* membrane vesicles were prepared in absence or presence of protease inhibitor cocktail Complete™ and, after SDS/PAGE, blotted onto poly(vinylidene difluoride) membranes. Immunoblots were developed with a polyclonal Ab directed against a synthetic peptide corresponding to a SecY domain (lanes 1 and 2), and a polyclonal Ab against SecA (lanes 3 and 4). SecY and SecA are indicated with an arrow, and the SecY proteolytic fragments are indicated by a bracket.

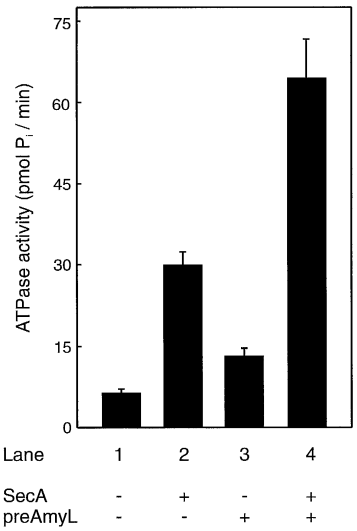

Fig. 3. Translocation ATPase assay. Urea-treated *B. subtilis* membrane vesicles were incubated in the presence of 2 mM ATP in the absence or presence of SecA and preAmyL at a concentration of 10 μg/ml each. After incubation for 30 min at 37°C, reactions were stopped by chilling on ice, and the amount of released inorganic phosphate was determined. Standard errors of the mean are of three experiments.

vesicles bearing intact SecY (data not shown). These data demonstrate that the *B. subtilis* SecY protein is highly susceptible to proteinase digestion, and that intact SecY can only be obtained when proteolysis is efficiently prevented by the use of a proteinase-deficient *B. subtilis* strain and a general proteinase inhibitor cocktail.

PreAmyL stimulates the SecA translocation ATPase activity. To assay whether purified His_6-tagged preAmyL (Fig. 1B) stimulates the ATP hydrolysis by SecA, membrane vesicles were extracted with urea to inactivate the endogenous SecA and F_0F_1-ATPases [33, 45], and incubated with ATP in the presence or absence of SecA and preAmyL (Fig. 3). The low level ATPase activity of the urea-treated membrane vesicles (Fig. 3, lane 1) was enhanced upon the addition of *B. subtilis* SecA (lane 2),

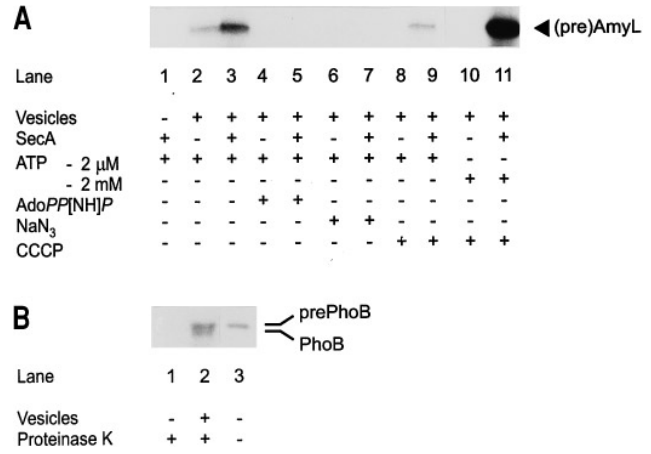

Fig. 4. Translocation and processing of precursor proteins by membrane vesicles of *B. subtilis* strain DB104. (A) Translocation of ^{125}I-preAmyL in the presence of 2 μM ATP as described in the Materials and Methods. As indicated, reactions were supplemented with Ado*PP*[NH]*P* (2 mM), sodium azide (15 mM), $CF_3OPhzC(CN)_2$ (10 μM) and purified *B. subtilis* SecA (10 μg/ml). (B) Translocation and processing of ^{125}I-prePhoB. Reactions were performed in the absence (lane 1) or presence (lane 2) of vesicles. After translocation, samples were treated with proteinase K and analysed. Lane 3 shows a 10% standard of the ^{125}I-prePhoB.

and further stimulated by preAmyL. These data demonstrate that the *B. subtilis* membrane vesicles support the preprotein-stimulated ATPase activity of SecA.

ATP and proton-motive-force-dependent translocation of preAmyL into *B. subtilis* membrane vesicles. For the translocation studies, preAmyL was iodinated with ^{125}I. Urea-denatured ^{125}I-preAmyL (about 5 nM final concentration) was subsequently diluted into a buffer containing native *B. subtilis* membrane vesicles, and translocation was initiated by the addition of MgATP. After 30 min incubation, translocation was assayed by the amount of proteinase-K-resistant ^{125}I-preAmyL using SDS/PAGE and autoradiography. In the presence of ATP, a considerable amount of the ^{125}I-preAmyL (about 10% added label) became protected for degradation by proteinase (Fig. 4A, lane 2). When the membrane vesicles (Fig. 4A, lane 1) or ATP (Fig. 5, lane 1) were omitted from the reaction, no proteinase resistant ^{125}I-preAmyL was observed. Obviously, the low amount of protease inhibitors still present in the translocation reaction does not interfere with the proteinase K activity. Translocation of preAmyL into the native membrane vesicles was even further stimulated by the addition of purified *B. subtilis* SecA protein (Fig. 4A, lane 3). When membrane vesicles were used prepared of strain 168, or of strain DB104 prepared without Complete™ were used, no proteinase-protected preAmyL could be detected (data not shown). These data demonstrate that the *B. subtilis* membrane vesicles are competent for preAmyL translocation.

To establish further the energy requirement for translocation, experiments were performed with non-hydrolysable ATP analogues and uncouplers that dissipate the proton-motive force. ATP hydrolysis is needed for the reaction, as no proteinase-protected preAmyL was observed when the ATP-dependent reaction was performed in the presence of an excess of the non-hydrolysable ATP analogue, adenosine 5′-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*) (Fig. 4A, lanes 4 and 5). At higher ATP concentration, i.e. 2 mM instead of 2 μM, Ado*PP*[NH]*P* was far less efficient as an inhibitor (data not shown). Protein export in *B. subtilis* strictly requires the proton motive force [16, 17]. The uncoupler (carbonylcyanide *p*-trifluoromethoxy-phenylhydra-

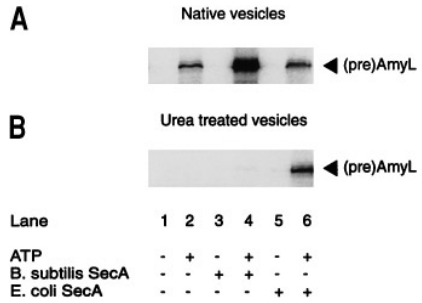

Fig. 5. Translocation of preAmyL is SecA dependent. ^{125}I-PreAmyL translocation reactions were performed with native (A) and urea-treated (B) membrane vesicles of *B. subtilis* strain DB104 as described in the Materials and Methods. As indicated, reactions were supplemented with purified *B. subtilis* or *E. coli* SecA (10 μg/ml) and ATP (2 mM).

zone) ($CF_3OPhzC(CN)_2$; 10 μM) effectively inhibited the ATP-dependent preAmyL translocation reaction (Fig. 4A, lane 8). An increased concentration of ATP could not relieve the inhibitory effect of $CF_3OPhzC(CN)_2$ (Fig. 4A, lane 10), but inhibition was efficiently prevented by an excess of purified *B. subtilis* SecA (lanes 9 and 11). These data suggest that the *in vitro* translocation reaction exhibits the same energetic requirements as *in vivo* protein export.

Processing of prePhoB. Due to the diffuse signal caused by the iodine label and the large size of the preAmyL (i.e. 58 kDa), it was not possible to resolve the precursor and mature forms of AmyL on SDS/PAGE. To demonstrate that the membranes actively process translocated precursor, translocation experiments were carried out with *B. subtilis* prePhoB (Fig. 4B). ^{125}I-PrePhoB efficiently translocates (about 30% of input prePhoB) into the *B. subtilis* inner membrane vesicles in an ATP-dependent manner (data not shown). About 40% of the translocated prePhoB was processed by signal peptidase to the mature PhoB that migrates with a higher mobility on SDS/PAGE (Fig. 4B, lane 2). The results demonstrate that the translocation activity of the isolated membranes is accompanied by processing of the signal sequence.

Translocation of preAmyL is SecA-dependent. Due to the presence of endogenous SecA, a strict SecA requirement is difficult to demonstrate with native membrane vesicles. Therefore, the effect of azide on the preAmyL translocation was determined. Azide efficiently blocks protein export in *E. coli* [38] and, at higher concentrations, also in *B. subtilis* [24, 46] cells. Azide blocks the translocation ATPase activity of SecA [38] and, by preventing hydrolysis of ATP, it traps the SecA in a membrane-inserted state [47]. Addition of 15 mM sodium azide completely inhibited the ATP-dependent translocation of preAmyL into *B. subtilis* membrane vesicles, both in the absence and presence of an excess of purified SecA protein (Fig. 4A, lanes 6 and 7). These data indirectly suggest that preAmyL translocation into *B. subtilis* membrane vesicles is SecA-dependent.

To establish directly the SecA requirement, experiments with performed with urea-treated membrane vesicles. This treatment inactivates membrane-bound SecA, and renders the translocation reaction in *E. coli* inner membrane vesicles dependent on the addition of purified SecA [33]. Urea treatment of *B. subtilis* vesicles completely abolished the translocation activity (compare Fig. 5A and B). However, addition of purified *B. subtilis* SecA to these membranes restored translocation only to a low level (Fig. 5B, lane 4). In contrast, addition of purified *E. coli* SecA resulted in a marked stimulation of translocation (Fig. 5B, lane 6), even though it had little effect on the translocation activity of non-urea-treated membrane vesicles (Fig. 5A). These data suggest that SecA is an essential component of the *B. subtilis* protein secretion apparatus, but that urea extracts or inactivates other factors that are needed to restore protein translocation to the level found for native membranes.

DISCUSSION

Due to the lack of a functional *in vitro* protein translocation system, studies on the mechanism of protein secretion in *B. subtilis* up to now have relied on *in vivo* pulse-chase experiments. With the *in vivo* techniques, translocation is usually assayed indirectly by the processing of the preprotein, while for the demonstration of true secretion, more elaborate proteolysis techniques have to be employed which include removal of the cell wall. Another approach of the *in vivo* studies is that due to the presence of extracellular proteases, degradation of the (partially) translocated proteins occurs [49−52]. We now have developed an *in vitro* system for preprotein translocation into *B. subtilis* membrane vesicles, capable of translocating and processing precursors with an efficiency comparable to that of the *E. coli* system [33]. This newly described system makes use of purified cytosolic components and preproteins and, thus, can now be used in a systematic manner for the analysis of the functioning and specificity of the translocase of *B. subtilis*, and its comparative analysis with the well-established *E. coli* translocase.

One major cause of the inability to functionally reconstitute preprotein translocation from *B. subtilis* membranes is the high susceptibility of SecY to proteases. Although we have not identified the proteolytic activity responsible for this degradation, it is likely caused by one or more of the secretory proteases. Strain DB104, which lacks the alkaline proteinase and which is deficient in neutral proteinase, still contains a substantial proteolytic activity. SecY was recovered in an intact form when the membrane vesicle isolation procedure was carried out in the presence of the Complete™ protease inhibitor cocktail. Complete™ is a commercial product with an unknown composition, but is particular active against endoproteinases. It is important to note that the Complete™ had to be used at the stage of resuspending the cells and onwards. SecA, the peripheral subunit of the translocase, was recovered in an intact form, irrespective of the use of Complete™ during the membrane isolation protocol. This method may also be useful for the study of other membrane-associated enzymes and processes such as solute transport.

The studies with the *B. subtilis* membrane vesicles now allow us to establish a number prominent features of the preprotein translocation reaction. Export of proteins from Bacilli has been shown to be sensitive to azide [17, 53] and uncouplers [16, 46], indicating that hydrolysis of ATP by SecA and the presence of a proton-motive force both are necessary for secretion from *B. subtilis*, as in the case in *E. coli*. This is also evident from the *in vitro* studies. Translocation strictly depends on the hydrolysis of ATP since no translocation is observed in the presence of the non-hydrolysable analog Ado*PP*[NH]*P*. Azide, a potent inhibitor of the translocation ATPase activity of SecA, also suppresses translocation, although high concentrations are needed to obtain complete inhibition. Possibly, at low azide concentration, slow hydrolysis of ATP by SecA still enables initiation of translocation. In conjunction with the proton motive force generated by the F_0F_1-ATPase, this low level of ATP hydrolysis may suffice efficient translocation. However, care should be taken with high concentrations of azide as this may also block the F_0F_1-ATPase, thereby causing a lowering of the proton-motive force. The low-level azide-resistant mutants of *E. coli* [38]

and *B. subtilis* [46, 53] are confined to the *secA* (*divA*) gene. In agreement with the *in vivo* studies [16], dissipation of the proton motive force inhibits translocation of preAmyL *in vitro*. As in *E. coli* [54], an excess of purified SecA can overcome this constraint. However, an excess of SecA is unable to compensate for the inhibition by azide. We have recently shown for *E. coli* SecA that azide interferes with the SecA catalytic cycle [47], and that it traps the SecYEG-bound SecA in the ATP-occupied conformational state, i.e. the membrane inserted state. Even in the absence of azide, SecA remains trapped in this state and cannot be exchanged for the free cytosolic form of SecA. Obviously, the inhibited SecA occupies the translocation sites and, thereby, prevents the initiation of new translocation events. The data on the azide inhibition of preAmyL translocation into *B. subtilis* membrane vesicles are consistent with a catalytic involvement of SecA.

Direct evidence of a requirement for SecA is provided by the studies with urea-treated vesicles. Urea removes and inactivates peripheral membrane proteins, and with *E. coli* inner membranes, translocation becomes dependent on the addition of purified SecA after urea treatment [33]. However, in order to restore preprotein translocation to the level observed with native membrane vesicles, the ability to generate a proton-motive force also has to be restored [55]. Urea treatment seems to have a similar effect on *B. subtilis* membrane vesicles. Translocation is completely abolished and can only be partially restored by the addition of purified *B. subtilis* SecA. Strikingly, the *E. coli* SecA restores translocation into urea-treated membranes to a substantial level. A mechanistic explanation for this phenomena cannot yet be given, and will require further investigations. For instance, it could be that the lack of a proton motive force is more harmful when the *B. subtilis* SecA is used than the *E. coli* protein. In *E. coli*, SecA has been shown to suppress the proton-motive-force dependency of preprotein translocation [54], but the exact mechanism has not yet been resolved. Studies with native membrane vesicles show that preAmyL translocation is strongly dependent on the proton-motive force. A more detailed study on the proton-motive force dependency of the translocation of preAmyL variants is in progress.

Our *in vitro* experiments do not strictly require the presence of a chaperone. A subset of preproteins in *E. coli* is dependent on the molecular chaperone SecB for *in vivo* and *in vitro* translocation [34, 56, 57]. Under certain conditions, other chaperones such as GroESL or DnaK/DnaJ/GrpE may support translocation [58−60]. However, unlike SecB, these chaperones have no targeting role. From *B. subtilis*, no data is available that would indicate that chaperones are needed to support protein secretion, and the *B. subtilis* chromosome does not appear to harbor a gene that codes for a SecB homologue which shows significant similarity to the *E. coli* enzyme [14]. When co-expressed in *B. subtilis*, *E. coli secB* supports the translocation of maltose-binding protein [61]. The SecB-binding domain of the *E. coli* SecA is confined to the carboxyl-terminal 22 amino acids [62]. This region, which is critical for the targeting function of SecB, is highly conserved among bacterial SecA proteins and is also present in the *B. subtilis* SecA. We have recently observed that the carboxyl-terminus of the *B. subtilis* SecA is able to bind SecB (Van Wely, K., unpublished results), consistent with the ability of *B. subtilis* SecA to support the SecB function *in vivo* [61] and *in vitro* (this data). Therefore, it will be important to determine if the carboxyl-terminus of SecA fulfills a rudimentary function or if it is involved in the binding of a cytosolic chaperone that has a similar function, but different structure to the *E. coli* SecB.

In conclusion, we have developed an *in vitro* system for the study of preprotein translocation in *B. subtilis.* Translocation of purified and urea-denaturated preAmyL is dependent on SecA and is driven by the hydrolysis of ATP and by the proton-motive force. The *in vitro* system opens a new area in the study of preprotein translocation in Bacilli and can be a useful tool to identify components of the protein-secretion machinery that, up to now, have been unknown in *B. subtilis*, such as functional homologues of the *E. coli* SecG and SecB, and possibly other novel components. These studies may provide new leads in the optimisation of the secretion of heterologous proteins, and will be useful for comparative purposes with the paradigm secretory system of *E. coli*.

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